Altered fibrin clot structure and dysregulated fibrinolysis contribute to thrombosis risk in severe COVID-19

Wygrecka, Malgorzata ; Birnhuber, Anna ; Seeliger, Benjamin ; Michalick, Laura ; Pak, Oleg ; Schultz, Astrid-Solveig ; Schramm, Fabian ; Zacharias, Martin ; Gorkiewicz, Gregor ; David, Sascha ; Welte, Tobias ; Schmidt, Julius J ; Weissmann, Norbert ; Schermuly, Ralph T ; Barreto, Guillermo ; Schaefer, Liliana ; Markart, Philipp ; Brack, Markus C ; Hippenstiel, Stefan ; Kurth, Florian ; Sander, Leif ; Witzenrath, Martin ; Kuebler, Wolfgang ; Kwapiszewska, Grazyna ; Preissner, Klaus T

Abstract: The high incidence of thrombotic events suggests a possible role of the contact system pathway in COVID-19 pathology. Here, we demonstrate altered levels of factor XII (FXII) and its activation products in critically ill COVID-19 patients in comparison to patients with severe acute respiratory distress syndrome due to influenza virus (ARDS-influenza). Compatible with this data, we report rapid consumption of FXII in COVID-19, but not in ARDS-influenza, plasma. Interestingly, the lag phase in fibrin formation, triggered by the FXII activator kaolin, was not prolonged in COVID-19 as opposed to ARDS-influenza. Using confocal and electron microscopy, we showed that increased FXII activation rate, in conjunction with elevated fibrinogen levels, triggers formation of fibrinolysis-resistant, compact clots with thin fibers and small pores in COVID-19. Accordingly, clot lysis was markedly impaired in COVID-19 as opposed to ARDS-infleunza subjects. Dysregulatated fibrinolytic system, as evidenced by elevated levels of thrombin-activatable fibrinolysis inhibitor, tissue-plasminogen activator, and plasminogen activator inhibitor-1 in COVID-19 potentiated this effect. Analysis of lung tissue sections revealed wide-spread extra- and intra-vascular compact fibrin deposits in COVID-19 patients. Together, compact fibrin network structure and dysregulated fibrinolysis may collectively contribute to high incidence of thrombotic events in COVID-19.

DOI: https://doi.org/10.1182/bloodadvances.2021004816

blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Altered fibrin clot structure and dysregulated fibrinolysis contribute to thrombosis risk in severe COVID-19

Tracking no: ADV-2021-004816R2

Malgorzata Wygrecka (University of Giessen Lung Center, Germany) Anna Birnhuber (Ludwig Boltzmann Institute for Lung Vascular Research, Austria) Benjamin Seeliger (Hannover Medical School, Germany) Laura Michalick (Charité - Universitaetsmedizin Berlin, Germany) Oleg Pak (Justus-Liebig University Giessen, Germany) Astrid-Solveig Schultz (University of Giessen Lung Center, Germany) Fabian Schramm (University of Giessen Lung Center, Germany) Martin Zacharias (Medical University Graz, Austria) Gregor Gorkiewicz (Medical University of Graz, Austria) Sascha David (University Hospital Zurich, Switzerland) Tobias Welte (Hannover Medical School, Germany) Julius Schmidt (Hannover Medical School, Germany) Norbert Weissmann (Justus-Liebig University Giessen, Germany) Ralph Schermuly (Justus-Liebig University Giessen, Germany) Guillermo Barreto (Université de Lorraine, CNRS, Laboratoire IMoPA, UMR 7365., France) Liliana Schaefer (Goethe University Frankfurt, Germany) Philipp Markart (Fulda Hospital, University Medicine Marburg, Germany) Markus Brack (Charité - Universitaetsmedizin Berlin, Germany) Stefan Hippenstiel (Charite'-Universitatsmedizin, Germany) Florian Kurth (Charite'-Universitatsmedizin, Germany) Leif Sander (Charité-Universitätsmedizin, Germany) Martin Witzenrath (Charité - Universitätsmedizin Berlin, Germany) Wolfgang Kuebler (Institute of Physiology, Charité Berlin, Germany, Germany) Grazyna Kwapiszewska (Ludwig Boltzmann Institute for Lung Vascular Research, Austria) Klaus Preissner (Justus-Liebig-University, Medical School, Germany)

Abstract:
The high incidence of thrombotic events suggests a possible role of the contact system pathway in COVID-19 pathology. Here, we demonstrate altered levels of factor XII (FXII) and its activation products in critically ill COVID-19 patients in comparison to patients with severe acute respiratory distress syndrome due to influenza virus (ARDS-influenza). Compatible with this data, we report rapid consumption of FXII in COVID-19, but not in ARDS-influenza, plasma. Interestingly, the lag phase in fibrin formation, triggered by the FXII activator kaolin, was not prolonged in COVID-19 as opposed to ARDS-influenza. Using confocal and electron microscopy, we showed that increased FXII activation rate, in conjunction with elevated fibrinogen levels, triggers formation of fibrinolysis-resistant, compact clots with thin fibers and small pores in COVID-19. Accordingly, clot lysis was markedly impaired in COVID-19 as opposed to ARDS-infleunza subjects. Dysregulatated fibrinolytic system, as evidenced by elevated levels of thrombin-activatable fibrinolysis inhibitor, tissue-plasminogen activator, and plasminogen activator inhibitor-1 in COVID-19 potentiated this effect. Analysis of lung tissue sections revealed wide-spread extra- and intra-vascular compact fibrin deposits in COVID-19 patients. Together, compact fibrin network structure and dysregulated fibrinolysis may collectively contribute to high incidence of thrombotic events in COVID-19.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: M.W. designed the study, performed experiments, analyzed data, and wrote the manuscript; A.B., L.M., and O.P. performed experiments and analyzed data; B.S., S.D., T.W., J.J.S., M.C.B., S.H., F.K., L.E.S., and M. Witzenrath recruited patients, analyzed patient clinical data, and reviewed the manuscript; A-S.S. and F.S. analyzed patient clinical data and wrote the manuscript; M.Z. and G.G. collected autopsy tissue samples and reviewed the manuscript; N.W., R.T.S., G.B., L.S., and P.M. analyzed data and contributed to the writing of the manuscript; W.M.K., G.K., and K.T.P designed the study and wrote the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data sharing requests should be sent to Malgorzata Wygrecka (e-mail: malgorzata.wygrecka@innere.med.uni-giessen.de)

Clinical trial registration information (if any):

Figure 1

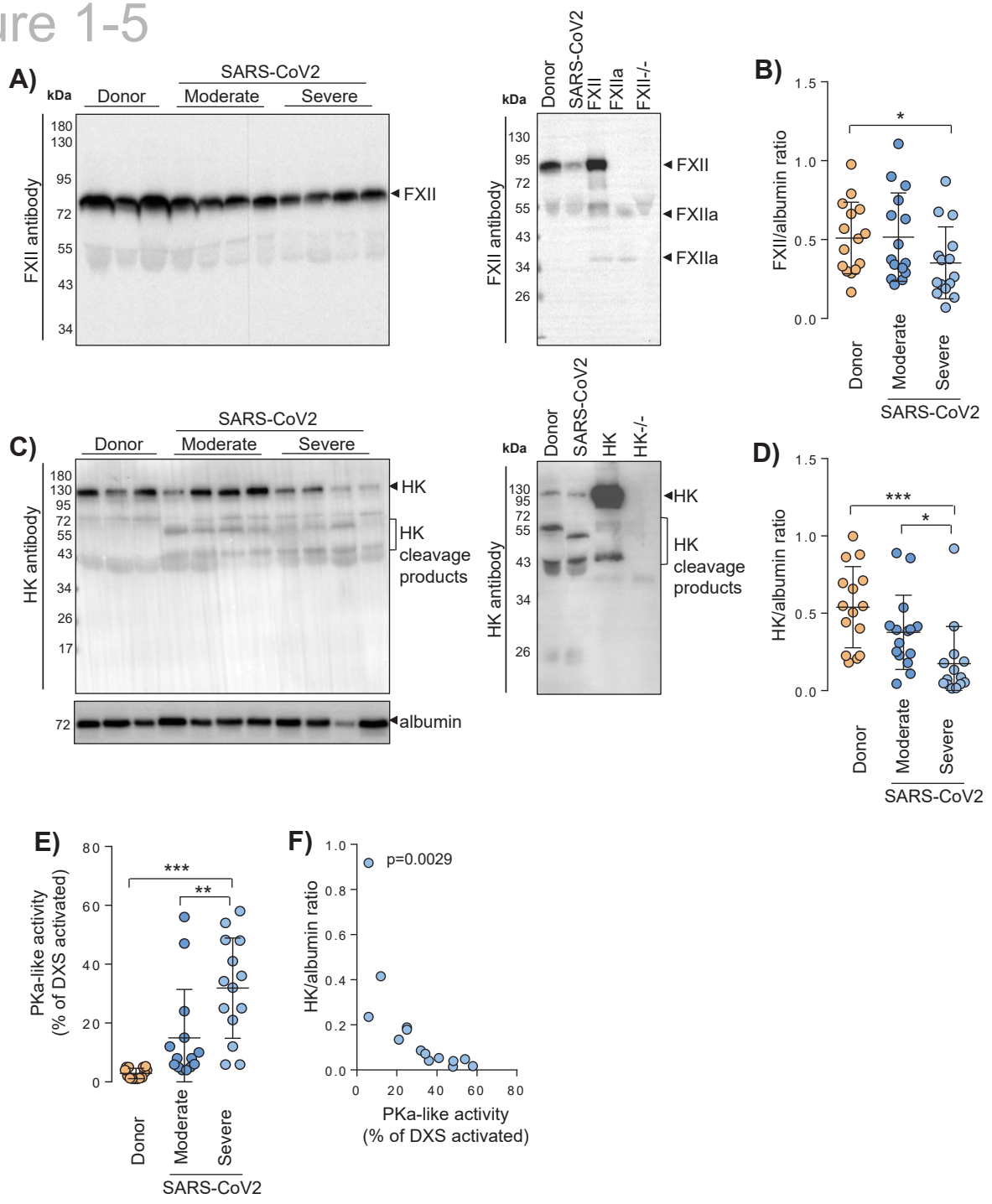


Figure 1-5

Figure 2

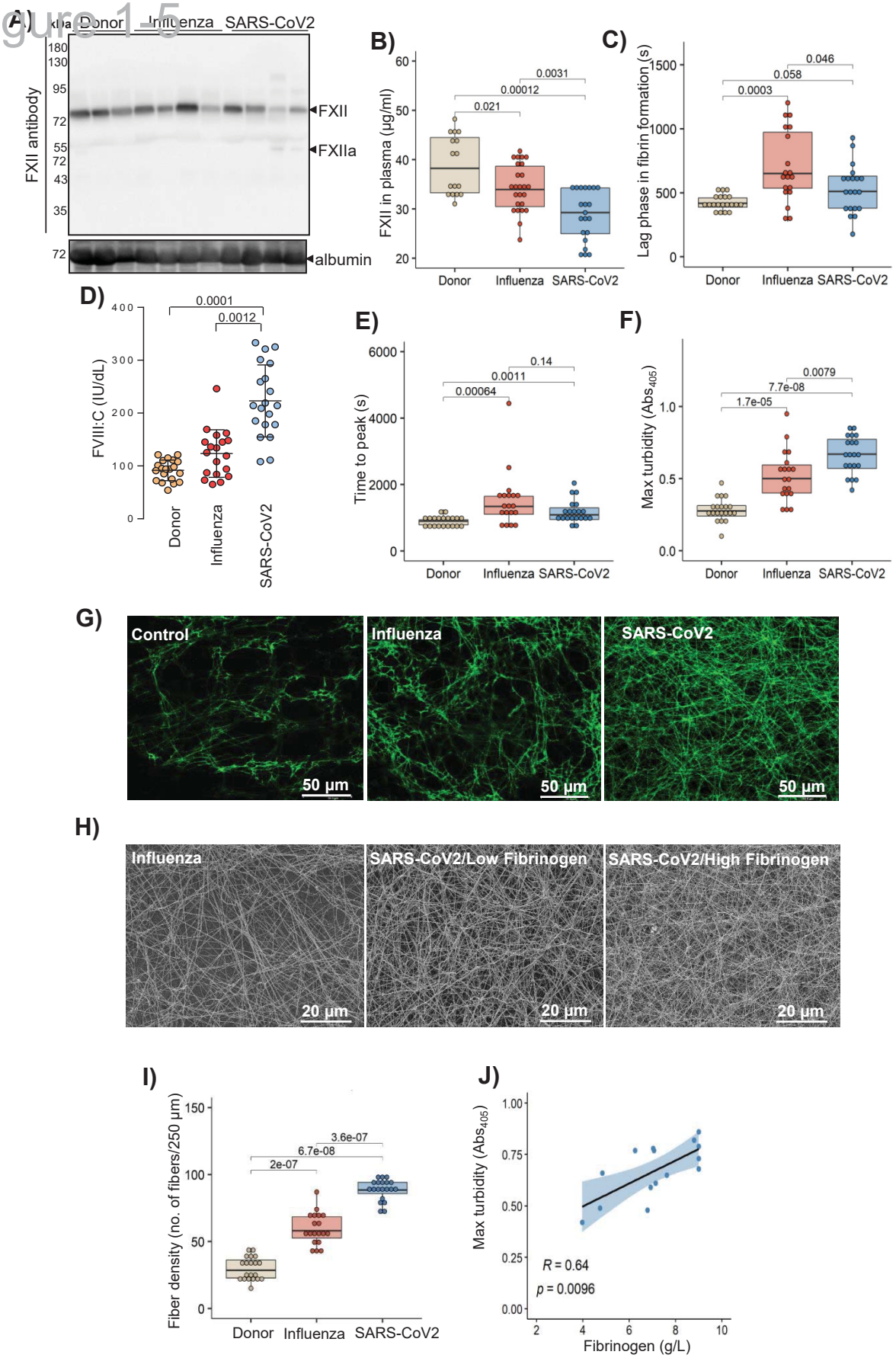


Figure 3

Figure 1-5

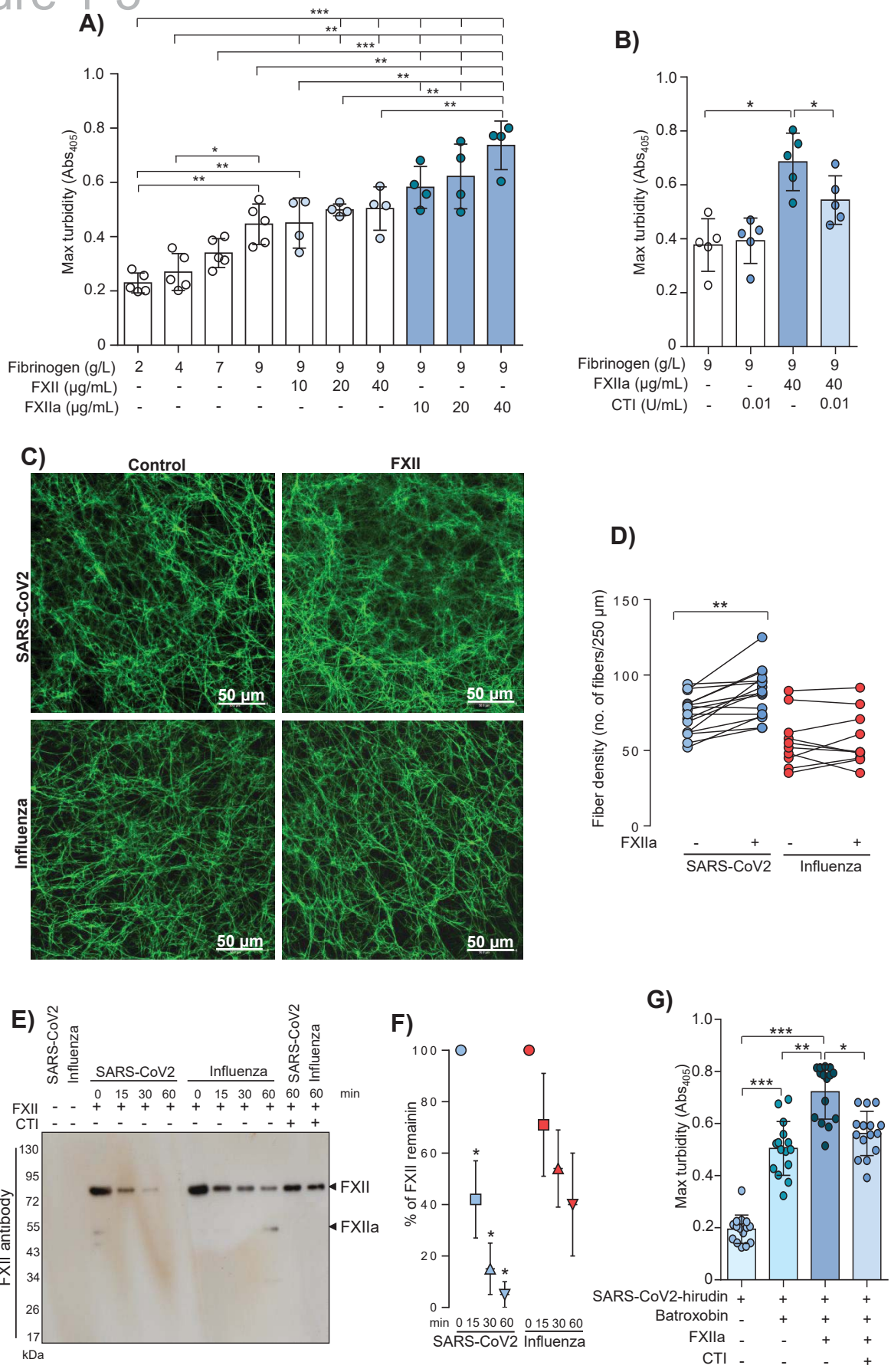


Figure 4

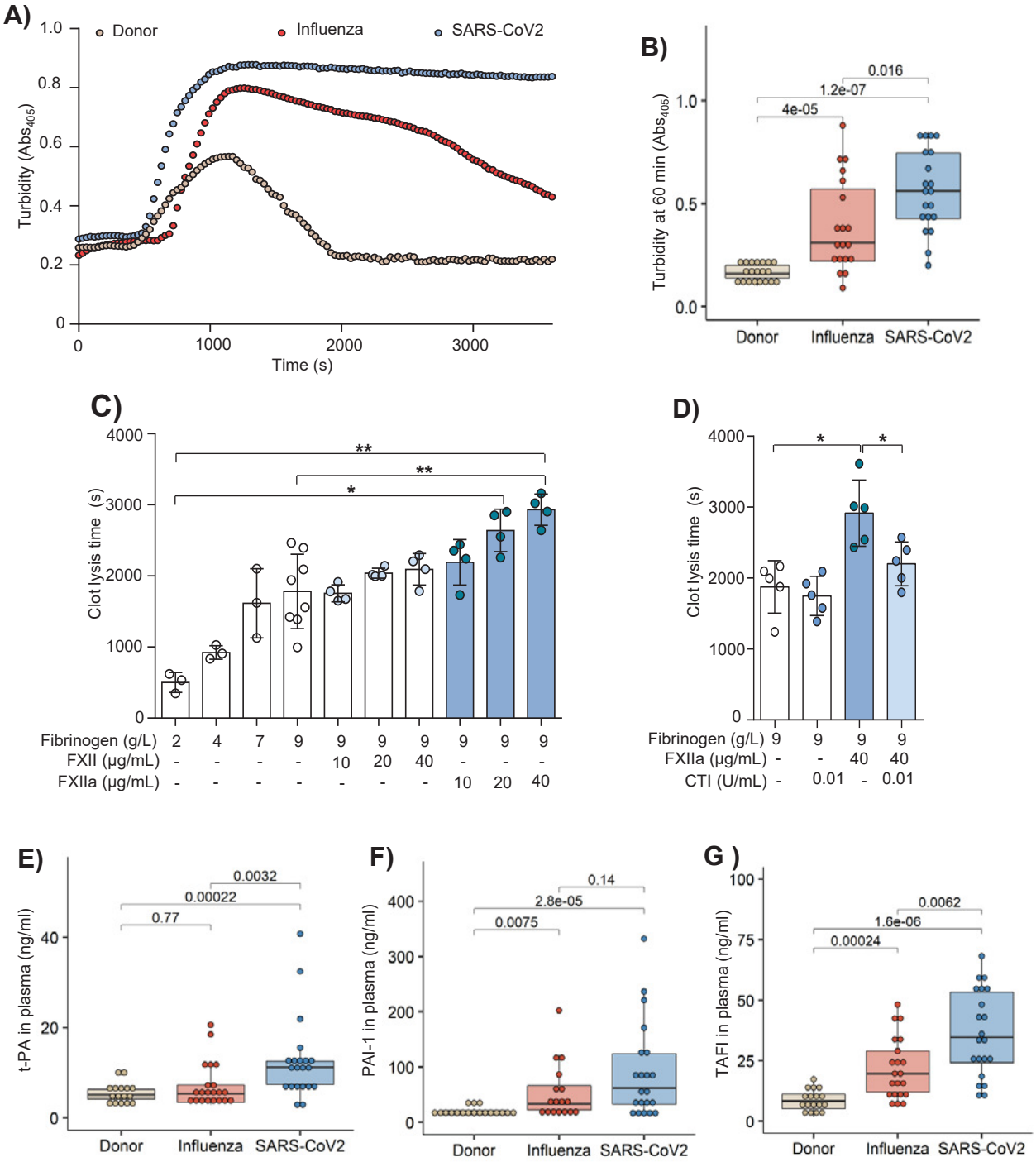
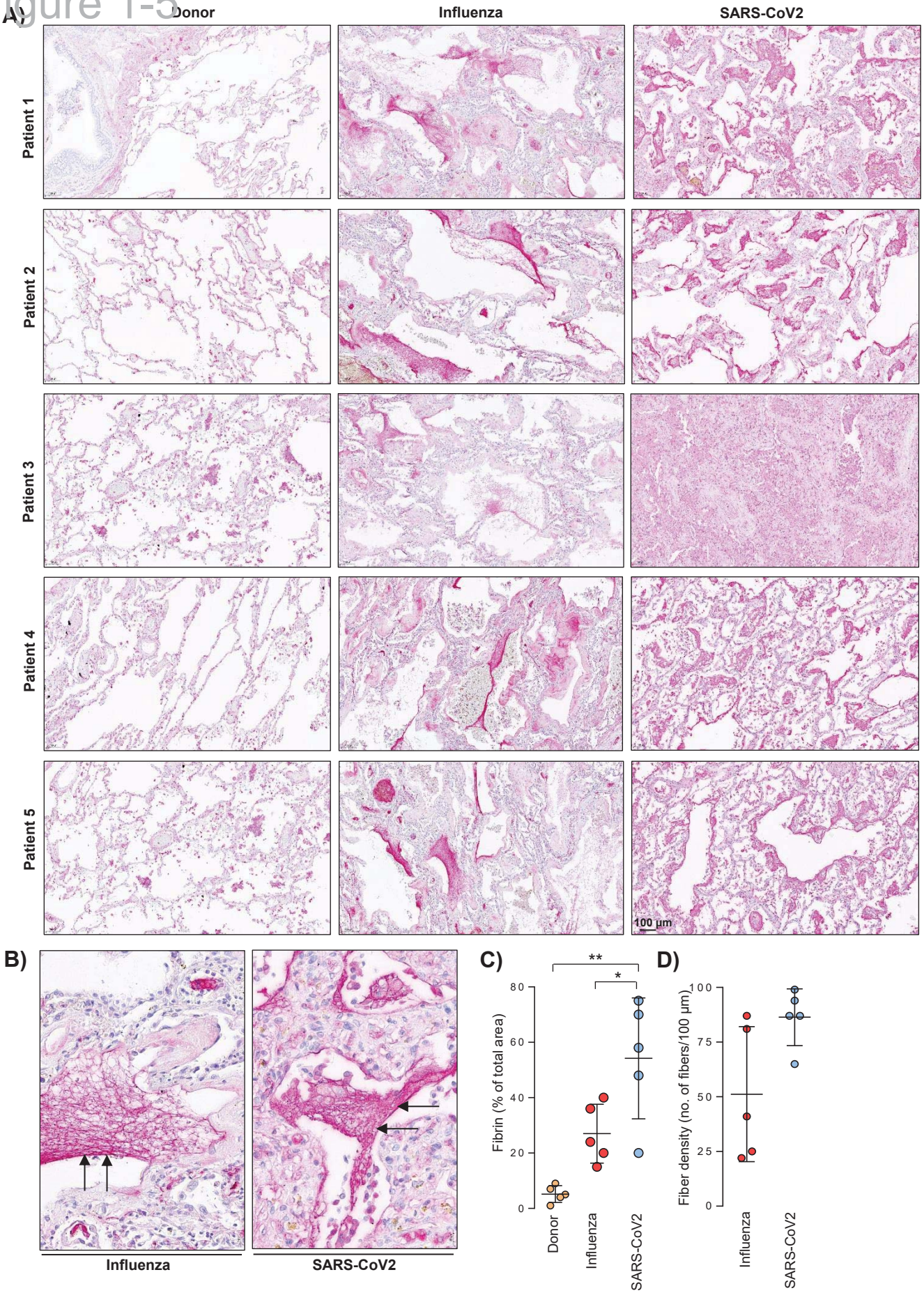


Figure 5

Figure 1-5



Altered fibrin clot structure and dysregulated fibrinolysis contribute to thrombosis risk in severe COVID-19

Malgorzata Wygrecka[1†], Anna Birnhuber[2], Benjamin Seeliger[3†], Laura Michalick[4], Oleg Pak[5], Astrid-Solveig Schultz[1], Fabian Schramm[1], Martin Zacharias[6], Gregor Gorkiewicz[6], Sascha David[7], Tobias Welte[3†], Julius J. Schmidt[8], Norbert Weissmann[5†], Ralph T. Schermuly[5†] Guillermo Barreto[9, 10†], Liliana Schaefer[11], Philipp Markart[12†], Markus C. Brack[13†], Stefan Hippenstiel[13†], Florian Kurth[13†], Leif E. Sander[13†], Martin Witzenrath[13†], Wolfgang M. Kuebler[4†], Grazyna Kwapiszewska[2], Klaus T. Preissner[14†].

[1]Center for Infection and Genomics of the Lung (CIGL), Universities of Giessen and Marburg Lung Center, Giessen, Germany. [2]Ludwig Boltzmann Institute for Lung Vascular Research, Medical University of Graz, Austria. [3]Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany. [4]Institute of Physiology, Charité-Universitätsmedizin, Berlin, Germany. [5]Excellence Cluster Cardiopulmonary Institute, Universities of Giessen and Marburg Lung Center, Giessen, Germany. [6]Diagnostic and Research Institute of Pathology, Medical University Graz, Austria. [7]Institute of Intensive Care, University Hospital Zurich, Zurich, Switzerland. [8]Department of Nephrology and Hypertension, Hannover Medical School, Hannover, Germany. [9]Paris-Est Creteil University, Gly-CRRET, Creteil, France. [10]Laboratoire IMoPA, UMR 7365 CNRS-Université de Lorraine, Biopôle de l'Université de Lorraine, Campus Biologie-Santé, Faculté de Médecine, Vandœuvre-lès-Nancy Cedex, France. [11]Institute of Pharmacology and Toxicology, Goethe University, Frankfurt Am Main, Germany. [12]Department of Pulmonary Medicine, Fulda Hospital, University Medicine Marburg, Campus Fulda, Fulda, Germany. [13]Department of Infectious Diseases and Respiratory Medicine, Charité Universitätsmedizin Berlin, Berlin, Germany. [14]Kerckhoff-Heart-Research-Institute, Department of Cardiology, Medical School, Justus-Liebig-University, Giessen, Germany. [†]Member of the German Center for Lung Research.

To whom correspondence should be addressed:
Malgorzata Wygrecka, PhD
Center for Infection and Genomics of the Lung (CIGL)
Universities of Giessen and Marburg Lung Center
Aulweg 132, 35392 Giessen, Germany
Phone: +49-641-99-36460; Fax: +49-641-99-36439
E-mail: malgorzata.wygrecka@innere.med.uni-giessen.de

Short title: Fibrin clot structure in COVID-19
Abstract word count: 193
Manuscript word count: 3961
Figure/table count: 5/2
Reference count: 62

Abstract

The high incidence of thrombotic events suggests a possible role of the contact system pathway in COVID-19 pathology. Here, we demonstrate altered levels of factor XII (FXII) and its activation products in critically ill COVID-19 patients in comparison to patients with severe acute respiratory distress syndrome due to influenza virus (ARDS-influenza). Compatible with this data, we report rapid consumption of FXII in COVID-19, but not in ARDS-influenza, plasma. Interestingly, the lag phase in fibrin formation, triggered by the FXII activator kaolin, was not prolonged in COVID-19 as opposed to ARDS-influenza. Using confocal and electron microscopy, we showed that increased FXII activation rate, in conjunction with elevated fibrinogen levels, triggers formation of fibrinolysis-resistant, compact clots with thin fibers and small pores in COVID-19. Accordingly, clot lysis was markedly impaired in COVID-19 as opposed to ARDS-infleunza subjects. Dysregulatated fibrinolytic system, as evidenced by elevated levels of thrombin-activatable fibrinolysis inhibitor, tissue-plasminogen activator, and plasminogen activator inhibitor-1 in COVID-19 potentiated this effect. Analysis of lung tissue sections revealed wide-spread extra- and intra-vascular compact fibrin deposits in COVID-19 patients. Together, compact fibrin network structure and dysregulated fibrinolysis may collectively contribute to high incidence of thrombotic events in COVID-19.

Key points

- Elevated fibrinogen, in conjunction with accelerated formation of FXIIa, may promote compact fibrin clot architecture in COVID-19.
- Dense fibrin network and dysregulated fibrinolysis collectively contribute to high incidence of thrombotic events in COVID-19.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a corona virus that causes a multisystem disease emanating from the respiratory tract designated as a coronavirus disease (COVID)-19[1–3]. Rapidly accumulating data suggests that a major underlying molecular mechanism in COVID-19-related morbidity and mortality is widespread endothelial injury associated with hyperactivation of the immune system, consequently leading to numerous haemostasis abnormalities[4-8].The clinical relevance of these processes is highlighted by the association between abnormal levels of D-dimer and the 28-day mortality in patients with COVID-19[9-13], and post-mortem studies stressing the presence of micro-thrombi and capillarostasis in the lungs of affected subjects[14,15].

The high incidence of thrombotic events, in particular deep vein thrombosis and pulmonary embolism, in conjunction with mildly prolonged activated partial thromboplastin time (APTT)[16,17], suggests a possible role of coagulation factor XII (FXII) in COVID-19 coagulopathy. FXII is a serine protease of the contact-phase system of blood coagulation which circulates in plasma as a single-chain zymogen[18]. Following contact with anionic surfaces such as kaolin, but also extracellular RNA (eRNA) released from damaged cells[19], neutrophil extracellular traps (NETs)[20], or polyphosphates secreted from activated platelets[21], FXII undergoes autoactivation to αFXIIa (herein referred to as FXIIa). FXIIa cleaves plasma prekallikrein (PK) to kallikrein (PKa), which in turn reciprocally activates FXII and amplifies FXIIa generation. As a consequence, the plasma kallikrein-kinin system is activated, leading to the release of the vasodilatory and vascular barrier disrupting peptide bradykinin from high molecular weight kininogen (HK)[22-25]. Overall, activation of the contact-phase system may contribute to an increased production of thrombin and fibrin, although FXIIa/PKa-mediated conversion of plasminogen to plasmin may also affect fibrinolysis[26].

A congenital deficiency of FXII in humans does not cause any bleeding complications, suggesting that FXII is dispensable for physiological haemostasis and fibrin formation[27]. However, the contact phase pathway may play an important role in thrombosis development when contact surfaces are exposed in scenarios such as trauma injury or bacterial and viral infections[27-29]. Indeed, numerous animal studies have confirmed a critical function of FXII in thrombus growth and stabilization under the mentioned conditions and provided the rationale for the development of new

FXIIa inhibitors, which ensure thrombo-protection in patients without causing a bleeding complications[27-30].

Given the high incidence of thromboembolic complications in severely ill COVID-19 patients[16,17], we investigated the contribution of FXII to clot formation and its architecture in this patient cohort in comparison to patients infected with the influenza virus.

Materials and Methods

Additional methods are provided in the supplement.

Study population

Plasma samples from COVID-19 patients were obtained from the Charité-University Medicine, Berlin, Germany (Berlin cohort) and from the Hannover Medical School, Hannover, Germany (Hanover cohort). Plasma samples from acute respiratory distress syndrome (ARDS) due to influenza were provided from the Hannover Medical School, Hannover, Germany. All samples were taken within 6 days after onset of ARDS. All investigations were approved by the local ethics committees (Hanover samples: SEPSIS/ARDS Registry 8146_BO_K_2018; Berlin samples: EA2/066/20) and written informed consent was obtained from all participants or their next-of-kin. COVID-19 patients were classified as moderate (hospitalized, no invasive ventilation; WHO severity score: 3-4) or severe (high flow O_2 or intubated and mechanically ventilated; WHO severity score: 5-7) as previously described[31]. Donor (healthy subjects) samples were provided by the Charité-University Medicine, Berlin, Germany (EA2/075/15) and from the Justus-Liebig University, Giessen, Germany (05/00). Blood was collected to the sodium citrate blood specimen collection tubes via standard venipuncture. All blood biospecimen were processed without a stabilizing reagent, at room temperature, within 5h of collection, and stored at -80°C. All plasmas were used in analyses of circulating proteins as well as coagulation and fibrinolytic activity except when insufficient plasma from an individual subject was available. Baseline demographics and clinical characteristics of the donors and patients are shown in Table 1.

Lung specimens were obtained from 10 ARDS patients (5 COVID-19, 5 influenza) and 5 donors by autopsy. Time from death to autopsy was matched. All

investigations were approved by the local ethics committees (Medical Faculty of Justus-Liebig University: 29/01 and Medical University of Graz: 32-362ex19/20) and written informed consent was obtained from all participants or their next-of-kin if required. Baseline demographics and clinical characteristics of lung tissue donors are shown in Table 2.

Plasma clot formation and lysis

Twenty µL of plasma were preincubated for 10min with 20µL of 0.1M imidazole buffer, pH7.4, and 20µL of 0.3mg/mL kaolin in a clear, flat-bottomed 96-well plate. Clotting was initiated by the addition of 20µL of 20mM $CaCl_2$ in the absence or presence of tissue plasminogen activator (t-PA) (25ng/mL final; Sekisui Diagnostics, Burlington, MA). Turbidity was monitored at 405nm (A_{405}) every 30s for 60min at 37°C using a SpectraMax 190 (Molecular Devices, Biberach, Germany). In some experiments, COVID-19 plasma was preincubated with hirudin (5IE/mL final; Diapharma, West Chester, OH) and the clotting was induced by batroxobin (5U/mL final; Enzyme Research Laboratories, South Bend, IN).

Fibrin formation and lysis in a purified system

Thrombin (5nM final, Sekisui Diagnostics) was mixed with fibrinogen (2-9g/L final, Thermo-Fisher Scientific, Waltham, MA), pre-incubated with either FXII or FXIIa (10-40µg/mL final, both from Sekisui Diagnostics) in a total volume of 25µL of 0.1M imidazole buffer in a clear, flat-bottomed 96-well plate. Fibrin formation was initiated by the addition of 20µL of 20mM $CaCl_2$. To measure fibrinolysis, t-PA (0.1µg/mL final) and plasminogen (20µg/mL final, Enzyme Research Laboratories) were added to the clotting solution. Turbidity was monitored as described above. In some experiments, FXIIa (40µg/mL final) was preincubated with corn trypsin inhibitor (CTI; 0.01U/mL final, Sekisui Diagnostics) before mixing with fibrinogen. FXII and FXIII contaminations were not detected in fibrinogen preparation by means of western blotting and ELISA.

FXII decay in plasma

Endogenous FXII was depleted from plasma using the goat anti-FXII antibody (cat. no.: 206-0056; Zytomed Systems) covalently attached to magnetic beads (Thermo-Fisher Scientific). Afterwards, a hundred µl of plasma was supplemented with 30nM

exogenous FXII and the sample was incubated for 1h at 37°C. Aliquots were withdrawn after the indicated time points and analyzed by western blotting. In some experiments, plasma was preincubated with 12mg/mL CTI 30min prior to the addition of FXII.

Activity assays

The PKa-like activity assay and the activity of factor VIII (FVIII:C) were performed as described in [32] and [33], respectively.

Statistics

Statistical analysis was performed in R (version 4) using the ggpubr package[34,35]. Data are expressed as single data points with boxplot overlay indicating median and interquartile range, unless indicated otherwise. Multiple groups were compared by non-parametric Kruskal-Wallis test. Correlations were performed using Spearman's rank correlation coefficient.

Data sharing statement

For original data, please contact malgorzata.wygrecka@innere.med.uni-giessen.de

Results

FXII is activated in severely ill COVID-19 patients

In the Berlin cohort, the plasma levels of FXII were decreased in severe COVID-19 patients as compared to donors (Figure 1A, B; moderate: WHO severity score 3–4; severe: WHO severity score 5–7). Disappearance of the FXII in plasma typically corresponds to its activation and conversion into the two chain FXIIa protein composed of the 50kDa heavy chain and 30kDa light chain. Detection of FXIIa in plasma is, however, hindered by its rapid inactivation and complex formation with C1 esterase inhibitor (C1INH). Thus to better monitor the presence of FXIIa in COVID-19 plasma, we monitored products of its activation, such as cleaved HK and PKa. As expected, disappearance of FXII in plasma was accompanied by HK cleavage, seen as diminished signal intensity of the intact HK band at 130kDa (Figure 1C, D). A decrease in intact HK levels was associated with the appearance of cleaved HK fragments: the cleaved HK light chain band migrating at 55kDa and an additional 45kDa band representing a degradation product of the 55kDa cleaved HK light chain. To further examine whether the reduction in intact levels of FXII and HK is a result of

the contact system activation, we measured the activity of plasma PKa. PKa-like activity was markedly elevated in severe COVID-19 patients in comparison to donors and patients with moderate SARS-CoV2 infection (Figure 1E). Furthermore, a strong negative correlation between the levels of intact HK/albumin ratio and the PKa-like activity in plasma of severe COVID-19 patients was observed (Figure 1F). Purified plasma proteins and deficient plasma samples were used to demonstrate the specificity of the bands shown in western blots (Figure 1A, C; right panels).

Fibrinogen and FXIIa regulate fibrin network density in COVID-19

To assess whether enhanced activation of FXII in critically ill COVID-19 patients represents a characteristic feature of SARS-CoV-2 infection, we analyzed plasma samples of patients with ARDS due to influenza virus infection. The decrease in FXII plasma levels in severe COVID-19 in comparison to donors was confirmed in the independent cohort (Hanover cohort) of the patients (Figure 2A). Furthermore, the levels of FXII in COVID-19 were significantly lower than those in ARDS-influenza (Figure 2B). Surprisingly yet, the lag phase in fibrin formation, triggered by the FXII activator kaolin, was shorter in plasma of COVID-19 patients as compared to patients with ARDS-influenza (Figure 2C). High plasma levels of FVIII:C in COVID-19 as opposed to ARDS-influenza provide one possible explanation for this effect (Figure 2D). Notably, both COVID-19 and ARDS-influenza patients received the same daily dose of unfractionated heparin, excluding iatrogenic anticoagulation as a cause of prolonged lag phase in fibrin formation in ARDS-influenza. In addition, we excluded lupus anticoagulant and the presence of anti-FXII antibodies as a cause of FXII deficiency in COVID-19 patients in our cohort (data not shown).

Further analysis of kaolin-triggered plasma clotting time revealed an increase in the time to reach the turbidity peak in both patient groups as compared to donors, but no difference between ARDS-influenza and COVID-19 (Figure 2E). The density of the clot (indicated by the maximum turbidity measurement) was higher in both patient groups as opposed to donors. A direct comparison between clots of ARDS-influenza and COVID-19 showed significantly higher maximal turbidity values in the latter group (Figure 2F). Visualization of fibrin clots by laser scanning confocal microscopy and scanning electron microscopy revealed an increase in fibrin structure compactness with thinner fibers and smaller pores in clots from COVID-19 plasma, as compared to clots generated from ARDS-influenza plasma (Figure 2G-I). A detailed analysis of the

clots generated from plasma of COVID-19 patients demonstrated association between packing density of fibrin fibers and plasma fibrinogen concentration, with dense fibrin network in clots formed in plasma of patients exhibiting high fibrinogen levels (Figure 2H). A strong positive correlation between maximum turbidity values and fibrinogen concentration in plasma of COVID-19 patients was noted (Figure 2J).

As the architecture of fibrin clots may be influenced not only by fibrinogen but also FXIIa[36,37], we next analyzed the impact of these two proteins on the clot structure in a purified system. As depicted in figure 3A high concentrations of fibrinogen increased peak turbidity values and this effect was potentiated by the addition of FXIIa. Accordingly, corn trypsin inhibitor (CTI), the inhibitor of FXIIa, reduced maximum turbidity of the clots generated by mixing fibrinogen and FXIIa (Figure 3B).

As sustained activation of FXII was described in COVID-19[38], we next investigated the potential contribution of FXIIa to the regulation of fibrin clot structure in this patient group. To this end, FXII-depleted COVID-19 and ARDS-influenza plasma samples were recalcified in the absence or presence of exogenous FXII and the fibrin clots were visualized using the antibody against fibrinogen/fibrin. As depicted in figure 3C and D, FXII increased fibrin network density but not fibrin fiber diameter in COVID-19 plasma. Yet, no apparent effect of FXII on the clot architecture in ARDS-influenza was observed (Figure 3C, D). Interestingly, the most prominent effect of FXII on fibrin network density was observed in COVID-19 plasma samples containing high levels of fibrinogen (Figure 3D). Together, these results imply that COVID-19 plasma contains FXII (auto)-activation cofactor(s) which trigger generation of FXIIa. FXIIa then affects the fibrin clot structure in a thrombin-dependent and/or thrombin-independent manner. In line with this assumption, rapid decay of exogenous FXII in COVID-19 plasma was observed (Figure 3E, F). CTI markedly delayed disappearance of FXII suggesting that auto-activation of FXIIa occurs in COVID-19 and ARDS-influenza plasma samples (Figure 3E). To demonstrate a direct, thrombin-independent, effect of FXIIa on the fibrin clot structure, we clotted hirudin-preincubated COVID-19 plasma with batroxobin in the presence of FXIIa and/or CTI and measured maximum turbidity. As shown in figure 3G, FXIIa increased maximum turbidity and this effect was diminished by CTI. These results are in line with the experiments performed in the purified system (Figure 3A, B). In sum, our results imply that FXIIa, in addition to its possible effect on thrombin generation, may also directly contribute to the fibrin network structure in COVID-19 plasma.

Elevated fibrin network density correlates with increased clot resistance to fibrinolysis

Compact fibrin network density was previously found to impair clot fibrinolysis[39]. Accordingly, we next evaluated the lysis resistance of fibrin clots in patient plasma, using an *in vitro* turbidimetric clot-lysis assay. Here, kaolin together with t-PA were added to plasma to initiate the intrinsic pathway of coagulation, followed by fibrin-dependent plasmin generation via t-PA-mediated activation of plasminogen in the same sample. While in donor plasma, the characteristic bell-shaped clot-lysis curve, representing the complete fibrin clot dissolution, was observed, only partial clot-lysis was detected in ARDS-influenza samples, and clot-lysis was absent in COVID-19 samples over the entire time period of the experiment (Figure 4A). This observation is supported by the highest turbidity values at 60min in COVID-19 samples (Figure 4B). Overall, clot lysis (as assessed by turbidity values at 60min) was observed in 84% of ARDS-influenza patients and only 30% of COVID-19 patients suggesting pronounced deregulation of the fibrinolytic system in SARS-CoV2-infected subjects in our cohort. As expected, increasing amounts of fibrinogen and FXIIa prolonged clot lysis time, with an additive effect being observed at the highest concentrations of both proteins (Figure 4C). The addition of CTI to the assay shortened clot lysis time (Figure 4D).

To test whether other components of the fibrinolytic system, such as t-PA, PAI-1 and TAFI, may be dysregulated in critically ill COVID-19 patients, we measured their levels by means of ELISA. The concentration of t-PA was elevated in COVID-19 as compared to donors and ARDS-influenza patients (Figure 4E). An increase of PAI-1 was also noted in plasma of ARDS-influenza and COVID-19 patients as opposed to donors, yet, a significant difference between both patient groups was not detected (Figure 4F). Interestingly, TAFI was not only markedly elevated in both patient groups as compared to donors, but it was also higher in patients with COVID-19 as compared to ARDS-influenza (Figure 4G).

Dense fibrin clots are observed in the lungs of severe COVID-19 patients

To demonstrate the *in vivo* relevance of our findings, we stained for fibrin the autopsy lung tissue sections from SARS-CoV2- and influenza-infected ARDS patients as well as subjects who died due to no respiratory causes. Notably, time from death to

autopsy was matched for all groups examined. As demonstrated in figure 5A, intra- and extra-vascular fibrin aggregates were observed in both severe COVID-19 and ARDS-influenza patients. However, in contrast to ARDS-influenza subjects, in the lungs of COVID-19 patients the deposits of fibrin appeared to be more widespread and evenly present not only in vascular but also alveolar spaces. In ARDS-influenza patients, fibrin deposit were predominantly observed in alveolar spaces and present in selected regions of the lung (Figure 5A, C). Overall, in COVID-19 lungs fibrin clots were more compact and homogeneous whereas in ARDS-influenza lungs they were widespread and characterized by regions of high and low fibrin fiber density (Figure 5B, D).

Discussion

Many patients with severe COVID-19 exhibit coagulation abnormalities that mimic other systemic coagulopathies associated with severe infections, such as disseminated intravascular coagulation (DIC) or thrombotic microangiopathy[40]. A high incidence of venous thromboembolism, pulmonary embolism, deep vein thrombosis, and multiple organ failure with a poor prognosis and outcome appears to be causally related to dysregulation of blood coagulation in critically ill COVID-19 patients. Besides an elevated inflammatory status that might induce monocyte-related coagulation and suppression of anticoagulant pathways, typical laboratory findings in COVID-19 patients are increased D-dimer levels and elevated fibrinogen concentrations[40]. Moreover, inflammation-induced endothelial cell injury in different vascular beds may contribute to a hypercoagulable state and the risk of thromboembolic complications[41,42].

In order to provide mechanistic insights into the reported hypercoagulable state of severe COVID-19 patients, we compared changes in the contact phase system activation and fibrinolysis between COVID-19 patients, individuals with ARDS-influenza, and donors (healthy subjects). While some critical parameters such as fibrinogen, t-PA, and TAFI were significantly increased, FXII levels were reduced in severe COVID-19, and the process of fibrin formation and the resulting fibrin clot structure and lysis were substantially different between patient cohorts. Histological data provided evidence for widespread, compact fibrin deposition in the lungs of patients with COVID-19 as opposed to those with ARDS-influenza.

In particular, the levels of FXII were decreased in severe COVID-19 patients as compared to ARDS-influenza and donors and FXII-activation products, such as cleaved HK and PKa-like activity, were altered in patients with SARS-CoV2 infection. This scenario very likely reflects FXII consumption due to its auto-activation on negatively charged surfaces and its reciprocal activation by PKa. Decreased FXII levels in COVID-19 plasma are also in accordance with moderately elevated APTT values reported in other studies[43,44]. The exacerbated consumption of FXII in severe COVID-19 is further supported by our *in vitro* studies, in which the supplementation of COVID-19 plasma with exogenous FXII resulted in its rapid activation, presumably due to the presence of FXII auto-activation cofactors or increased PKa activity. Indeed, common pathological events observed in COVID-19 such as increased tissue cell stress together with virus-mediated necrosis, endothelial dysfunction, and excessive neutrophil activation, lead to the release/exposure of large amounts of negatively charged molecules including NETs. NETs not only bind FXII but also serve as a potent endogenous inflammation-dependent inducer of FXII auto-activation, eventually propagating thrombosis[45]. Enhanced vascular NETosis along with impaired NET clearance were described in COVID-19 patients[38,46]. In line with these findings, several studies found an increase in NET components in COVID-19 plasma including cell-free DNA, myeloperoxidase-DNA complexes, neutrophil elastase-DNA complexes, and citrullinated histone H3[47,48]. In addition, active FXII was described to colocalized with NETs in the lungs of COVID-19 patients and NET positive pulmonary vessels were reported to be frequently clogged[38,49]. Together with these findings, our results speak for NET-induced, accelerated, and constant activation of FXII in COVID-19 and thus for its role in immunothrombothic processes in this pathological condition. In fact, FXII auto-activation cofactors were found to be relevant for the initiation and progression of sepsis and DIC[50, 51].

Interestingly enough, low plasma levels of FXII in severe COVID-19 patients did not result in the prolonged lag phase in fibrin formation, triggered by kaolin, in COVID-19 as opposed to ARDS-influenza. High levels of FVIII:C in the former group of the patients may provide one possible explanation for this effect. However, further studies assessing the levels of other coagulation factors such as factor IX and XI and their inhibitors as well as thrombin-antithrombin complexes are needed to thoroughly characterize high procoagulant activity of COVID-19 plasma. In particular, that Bouck *et al.* recently reported high thrombin generation potential of COVID-19 as opposed

to sepsis plasma[52]. These results, together with previously described high levels of fibrinogen, mild thrombocytopenia, and slightly altered plasma concentrations of coagulation factors and physiological anticoagulants argue for a specific form of intravascular coagulation in severe COVID-19 that is distinguishable from classical DIC seen in sepsis[53]. The prominent increase in vascular complications points to strong involvement of endothelial cells in hemostatic abnormalities seen in COVID-19. Injured endothelial cells may provide a scaffold for thrombus generation and elevated levels of von Willebrand factor multimers (recently described in COVID-19 plasma[54]) may facilitate platelet-vessel wall interactions ultimately leading to the formation of platelet-rich thrombotic deposits in microvasculature. Such platelet-rich thrombotic aggregates have been observed in alveolar capillaries of critically ill COVID-19 patients[16,17]. Altogether haemostatic alterations seen in COVID-19 subjects reflect widespread occlusive thrombotic microangiopathy with destruction of alveoli that supports persistence of microthrombi.

Increased levels of fibrinogen and elevated thrombin generation potential were found to contribute to the formation of stable clots that are composed of a dense network of the thin fibrin strands[36,55]. Accordingly, clots generated in COVID-19 plasma exhibited higher packing density and were more resistant to lysis as compared to clots formed in ARDS-influenza plasma. Further experiments with COVID-19 plasma revealed that next to fibrinogen also FXIIa may regulate clot compactness. While, increased fibrinogen levels can independently promote thrombus formation and stability[55], the role of FXII in this process seems to be more complex and dependent on the presence of cofactors, which enable FXII (auto)-activation. FXIIa may then regulate fibrin network density in a thrombin-dependent and a thrombin-independent manner. While, the ability of FXIIa to convert factor XI (FXI) to FXIa and thereby to promote thrombin generation is well documented[56,57], elucidation of the mechanism of a direct effect of FXIIa on fibrin clot architecture requires further research. FXIIa binds with high affinity to fibrinogen/fibrin[36], whether this interaction facilitates fibrin fiber cross-linking or incorporation of other components into a clot is speculative at the movement. Though, high turbidity of fibrin clots observed in COVID-19 may speak for the intercalation of, e.g. NET components, into the fibrin network. Cell free-DNA and histones (both NET components) were found to promote more opaque and fibrinolysis resistant clots[58,59]. In addition, cell free-DNA was reported to bind to fibrinogen, fibrin, FXII, FXIIa, HK, FIXa, FXIa, fibronectin, and vWF[58,60-62]. Thus, its

intercalation into fibrin network could not only accelerate FXII auto-activation but also serve as a platform that brings plasma-proteins and fibrin fibers together resulting in the formation of turbid and fibrinolysis resistant clots.

The persistent vessel occlusion, seen in critically ill COVID-19 patients, seems to be reinforced by markedly increased plasma levels of TAFI and moderately increased amounts of PAI-1. Elevated levels of t-PA try to counterbalance this prothrombotic environment, however, are not sufficient to compensate for increased procoagulant activity in patients with COVID-19. These findings are supported by recently published results showing that the enhanced thrombin generation potential is not adequately offset by increased plasmin generation potential in COVID-19[52]. High levels of fibrinogen and accelerated rate of FXIIa formation seem to potentiate this effect.

Our study has several limitations. First, the number of patients with COVID-19 and patients with ARDS-influenza is small and samples were collected at a single time point. Second, most of the patients with COVID-19 and patients with ARDS-influenza were receiving anticoagulant therapy. Five of the ARDS-influenza patients and seven of the patients with COVID-19 did not receive anticoagulation. However, we did not observe any differences in the parameters measured in the patients with or without anticoagulation. Third, although demographics and baseline laboratory values of ARDS-influenza and COVID-19 patients are comparable, there are some significant differences, e.g. in CRP or procalcitonin values, which might have an impact on our results. Yet, exclusion of the ARDS-influenza cases with the highest CRP or procalcitonin values did not change our results. Fourth, blood samples used in this study were not supplemented with contact phase pathway inhibitors, therefore, changes in the activation status of the contact phase proteins during blood sampling cannot be excluded. However, all samples were collected in the same manner. Finally, the measurements of plasma function in ARDS-influenza and COVID-19 were performed *in vitro*, hence, they may not fully reflect the processes occurring *in vivo*. Thus, further mechanistic studies *ex vivo* and *in vivo* are needed to fully elucidate the procoagulant and fibrinolytic changes in ARDS-influenza and COVID-19.

In conclusion, pathological events described in COVID-19 create milieu favoring activation of FXII. In combination with high levels of fibrinogen, FXIIa may contribute

to compact, lysis resistant clot formation in a thrombin-dependent and a thrombin-independent manner. This prothrombogenic microenvirement is further promoted by dysregulated fibrinolysis. Our study advances understanding of the common and divergent aspects related to clot formation and lysis during ARDS-influenza and COVID-19.

Acknowledgement

We thank E. Bieniek for her excellent technical assistance. We also thanks A. Seipp (Institute of Anatomy and Cell Biology, Justus Liebig University, Giessen, Germany) for performing the scanning electron microscopy pictures. This study was funded by the German Research Foundation (DFG: SFB/TR84 Project A2 to M.W. and W.M.K.), the Else Kröner-Fresenius-Foundation (2014_A179 to M.W. and P.M.), the Oskar Helene Heim Foundation (to P.M.), the German Center for Lung Research (82 DZL 005A1 to M.W.) and the University Medical Center Giessen and Marburg (UKGM to M.W. and P.M.).

Authorship

Contribution: M.W. designed the study, performed experiments, analyzed data, and wrote the manuscript; A.B., L.M., and O.P. performed experiments and analyzed data; B.S., S.D., T.W., J.J.S., M.C.B., S.H., F.K., L.E.S., and M. Witzenrath recruited patients, analyzed patient clinical data, and reviewed the manuscript; A-S.S. and F.S. analyzed patient clinical data and wrote the manuscript; M.Z. and G.G. collected autopsy tissue samples and reviewed the manuscript; N.W., R.T.S., G.B., L.S., and P.M. analyzed data and contributed to the writing of the manuscript; W.M.K., G.K., and K.T.P designed the study and wrote the manuscript.

Competing interests: None declared.

References

1. Gorbalenya A, Baker S, Baric R, et al. Severe acute respiratory syndrome-related coronavirus: The species and its viruses – a statement of the Coronavirus Study Group. *bioRxiv*. 2020;1–15.
2. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395(10223):497–506.
3. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020;579:265–269.
4. McFadyen JD, Stevens H, Peter K. The Emerging Threat of (Micro)Thrombosis in COVID-19 and Its Therapeutic Implications. *Circ. Res.* 2020;127(4):571–587.
5. Varga Z, Flammer AJ, Steiger P, et al. Endothelial cell infection and endotheliitis in COVID-19. *Lancet*. 2020;395(10234):1417–1418.
6. D'Alessandro A, Thomas T, Dzieciatkowska M, et al. Serum Proteomics in COVID-19 Patients: Altered Coagulation and Complement Status as a Function of IL-6 Level. *J. Proteome Res.* 2020;19(11):4417–4427.
7. Ibañez C, Perdomo J, Calvo A, et al. High D dimers and low global fibrinolysis coexist in COVID19 patients: what is going on in there? *J. Thromb. Thrombolysis*. 2020;1–5.
8. Wright FL, Vogler TO, Moore EE, et al. Fibrinolysis Shutdown Correlation with Thromboembolic Events in Severe COVID-19 Infection. *J. Am. Coll. Surg.* 2020;231(2):193–203.
9. Medcalf RL, Keragala CB, Myles PS. Fibrinolysis and COVID-19: A plasmin paradox. *J. Thromb. Haemost.* 2020;18(9):2118–2122.
10. Keragala CB, Medcalf RL, Myles PS. Fibrinolysis and COVID-19: A tale of two sites? *J. Thromb. Haemost.* 2020;18(9):2430–2432.
11. Sakka M, Connors JM, Hékimian G, et al. Association between D-Dimer levels and mortality in patients with coronavirus disease 2019 (COVID-19): a systematic review and pooled analysis. *JMV-Journal Med. Vasc.* 2020;45(5):268–274.
12. Seheult JN, Seshadri A, Neal MD. Fibrinolysis Shutdown and Thrombosis in Severe COVID-19. *J. Am. Coll. Surg.* 2020;231(2):203–204.
13. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J. Thromb. Haemost.* 2020;18(4):844–847.
14. Luo W, Yu H, Gou J-Z, et al. Clinical Pathology of Critical Patient with Novel Coronavirus Pneumonia (COVID-19): Pulmonary Fibrosis and Vascular Changes including Microthrombosis Formation. *Transplantation*. 2020;1–19.
15. Tian S, Xiong Y, Liu H, et al. Pathological study of the 2019 novel coronavirus disease (COVID-19) through postmortem core biopsies. *Mod. Pathol.* 2020;33:1007–1014.

16. Middeldorp S, Coppens M, van Haaps TF, et al. Incidence of venous thromboembolism in hospitalized patients with COVID-19. *J. Thromb. Haemost.* 2020;18(8):1995–2002.

17. Nahum J, Morichau-Beauchant T, Echegut P, et al. Venous Thrombosis Among Critically Ill Patients With Coronavirus Disease 2019 (COVID-19). *JAMA Netw. Open*. 2020;3(5):e2010478.

18. Weidmann H, Heikaus L, Long AT, et al. The plasma contact system, a protease cascade at the nexus of inflammation, coagulation and immunity. *Biochim. Biophys. Acta - Mol. Cell Res.* 2017;1864:2118–2127.

19. Kannemeier C, Shibamiya A, Nakazawa F, et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. *Proc. Natl. Acad. Sci. U. S. A.* 2007;104(15):6388–6393.

20. von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J. Exp. Med.* 2012;209(4):819–835.

21. Müller F, Mutch NJ, Schenk WA, et al. Platelet Polyphosphates Are Proinflammatory and Procoagulant Mediators In Vivo. *Cell*. 2009;139:1143–1156.

22. Kaplan AP, Meier HL, Mandle RJ. The role of Hageman factor, prekallifrein, and high molecular weight kininogen in the generation of bradykinin and the initiation of coagulation and fibrinolysis. *Monogr. Allergy*. 1977;12:120–130.

23. Müller F, Renné T. Novel roles for factor XII-driven plasma contact activation system. *Curr. Opin. Hematol.* 2008;15(5):516–521.

24. Kitamura N, Kitagawa H, Fukushima D, et al. Structural organization of the human kininogen gene and a model for its evolution. *J. Biol. Chem.* 1985;260(14):8610–8617.

25. Kitamura N, Nawa H, Takagaki Y, Furuto-Kato S, Nakanishi S. Cloning of cDNAs and Genomic DNAs for High-Molecular-Weight and Low-Molecular-Weight Kininogens. *Methods Enzymol.* 1988;163:230–240.

26. Braat EA, Dooijewaard G, Rijken DC. Fibrinolytic properties of activated FXII. *Eur J Biochem.* 1999;263(3):904-911.

27. Kleinschnitz C, Stoll G, Bendszus M, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J. Exp. Med.* 2006;203(3):513–518.

28. Hagedorn I, Schmidbauer S, Pleines I, et al. Factor XIIa Inhibitor Recombinant Human Albumin Infestin-4 Abolishes Occlusive Arterial Thrombus Formation Without Affecting Bleeding. *Circulation*. 2010;121(13):1510–1517.

29. Herwald H, Mörgelin M, Olsén A, et al. Activation of the contact-phase system on bacterial surfaces--a clue to serious complications in infectious diseases. *Nat Med.* 1998;4(3):298-302.

30. Worm M, Köhler EC, Panda R, et al. The factor XIIa blocking antibody 3F7: A safe anticoagulant with anti-inflammatory activities. *Ann. Transl. Med.* 2015;3(17):247.

31. Michalick L, Weidenfeld S, Grimmer B, et al. Plasma mediators in patients with severe COVID-19 cause lung endothelial barrier failure. Eur. Respir. J. 2020;56(6).

32. Zamolodchikov D, Chen ZL, Conti BA, Renné T, Stricklan S. Activation of the factor XII-driven contact system in Alzheimer's disease patient and mouse model plasma. Proc. Natl. Acad. Sci. U. S. A. 2015;112(13):4068–4073

33. Rauch A, Labreuche J, Lassalle F, et al. Coagulation biomarkers are independent predictors of increased oxygen requirements in COVID-19. J. Thromb. Haemost. 2020;18(11):2942–2953.

34. Alboukadel Kassambara (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. https://CRAN.R-project.org/package=ggpubr

35. H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

36. Konings J, Govers-Riemslag JWP, Philippou H, et al. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood*. 2011;118(14):3942–3951.

37. Machlus KR, Cardenas JC, Church FC, Wolberg AS. Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis in mice. *Blood*. 2011;117(18):4953–4963.

38. Englert H, Rangaswamy C, Deppermann C, et al. Defective NET clearance contributes to sustained FXII activation in COVID-19-associated pulmonary thrombo-inflammation. *EBioMedicine.* 2021;67:103382.

39. J.-P. Collet, J. Soria, M. Mirshahi, et al. Dusart syndrome: a new concept of the relationship between fibrin clot architecture and fibrin clot degradability: hypofibrinolysis related to an abnormal clot structure. *Blood*. 1993; 82(8):2462-2469

40. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. *Lancet Haematol.* 2020;7:E438–E440.

41. Klok FA, Kruip MJHA, van der Meer NJM, et al. Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: An updated analysis. *Thromb. Res.* 2020;191:148–150.

42. Birnhuber A, Fliesser E, Gorkiewicz G, et al. Between inflammation and thrombosis - endothelial cells in COVID-19. *Eur Respir J*. 2021;2100377.

43. Han H, Yang L, Liu R, et al. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. *Clin. Chem. Lab. Med.* 2020;58(7):1116–1120.

44. Harenberg J, Favaloro E. COVID-19: Progression of disease and intravascular coagulation-present status and future perspectives. *Clin. Chem. Lab. Med.* 2020;58(7):1029–1036.

45. Massberg S, Grahl L, Von Bruehl ML, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat. Med.* 2010;16(8):887–896.

46. Masso-Silva JA, Moshensky A, Lam MTY, et al. Increased peripheral blood

neutrophil activation phenotypes and NETosis in critically ill COVID-19 patients: a case series and review of the literature. *Clin Infect Dis*. 2021;ciab437.

47. Leppkes M, Knopf J, Naschberger E, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. *EBioMedicine* 2020;58:102925.

48. Veras FP, Pontelli MC, Silva CM, et al. SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J Exp Med* 2020;217(12):e20201129.

49. Zuo Y, Zuo M, Yalavarthi S, et al. Neutrophil extracellular traps and thrombosis in COVID-19. *J Thromb Thrombolysis*. 2020;1–8.

50. Popescu NI, Silasi R, Keshari RS, et al.Peptidoglycan induces disseminated intravascular coagulation in baboons through activation of both coagulation pathways. *Blood.* 132(8):849-860.

51. Silasi R, Keshari RS, Regmi G, et al. Factor XII plays a pathogenic role in organ failure and death in baboons challenged with Staphylococcus aureus. *Blood.* 2021;138(2):178-189.

52. Bouck EG, Denorme F, Holle LA, et al. COVID-19 and Sepsis Are Associated With Different Abnormalities in Plasma Procoagulant and Fibrinolytic Activity. *Arterioscler Thromb Vasc Biol.* 2021;41(1):401-414.

53. Iba T, Levy JH, Levi M, and Thachil J. Coagulopathy in COVID-19. *J Thromb Haemost.* 2020;18(9):2103-2109.

54. Ward SE, Fogarty H, Karampini E, et al. ADAMTS13 regulation of VWF multimer distribution in severe COVID-19. *J Thromb Haemost.* 2021 May 30.

55. Shah GA, Nair CH, Dhall DP.Physiological studies on fibrin network structure. *Thromb Res.* 1985;40(2):181-188.

56. Kurachi K, Davie EW. Activation of human factor XI (plasma thromboplastin antecedent) by factor XIIa (activated Hageman factor). *Biochemistry.* 1977;16(26):5831-5839.

57. Walsh PN.Roles of platelets and factor XI in the initiation of blood coagulation by thrombin. *Thromb Haemost.* 2001;86(1):75-82.

58. Gould TJ, Vu TT, Stafford AR, et al. Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis. *Arterioscler Thromb Vasc Biol*. 2015; 35(12):2544-2553.

59. Varju I, Longstaff C, Szabo L, et al. DNA, histones and neutrophil extracellular traps exert anti-fibrinolytic effects in a plasma environment. *Thromb Haemost*. 2015;113(6):1289–1298.

60. Vu TT, Leslie BA, Stafford AR, et al. Histidine-rich glycoprotein binds DNA and RNA and attenuates their capacity to activate the intrinsic coagulation pathway. *Thromb Haemost.* 2016;115(1):89-98.

61. Pande H, Calaycay J, Hawke D, Ben-Avram CM, Shively JE. Primary structure of a glycosylated DNA-binding domain in human plasma fibronectin. *J Biol Chem.* 1985;260(4):2301-2306.

62. Sandoval-Pérez A, Berger RML, Garaizar A, et al. DNA binds to a specific site

of the adhesive blood-protein von Willebrand factor guided by electrostatic interactions. *Nucleic Acids Res.* 2020;48(13):7333-7344.

Table 1. Baseline demographics and clinical characteristics of donors and COVID-19 and ARDS-influenza patients (plasma samples).

	Hanover cohort			Berlin cohort		
	ARDS[1]-influenza	COVID[2]-19 (WHO 5-7)	Donor	COVID-19 (WHO 5-7)	COVID-19 (WHO 3-4)	Donor
No. of patients, n	25	21	21	15	15	15
Age, year	56;[20-86]	59;[19-82]	60;[20-79]	61;[22-84]	61;[26-80]	61;[24-82]
Sex, male (%)	87	90	88	69	67	68
BMI (kg/m^2)	25;[20-36]	29 [15-62]	26;[19-45]	29;[25-36]	24;[20-36]	25;[22-37]
[3]CRB65 score, n						
0	0	0		NA	NA	
1	3	2				
2	5	6				
3	12	9				
4	5	4				
28-day mortality (%)	30	14.3		8	0	
[4]LOS ICU, days	19;[6-73]	27;[3-63]		30;[5-220]	NA	
Ventilation, days	15;[3-66]	16;[4-50]		26;[5-220]	NA	
[5]ECMO (%)	30	24		46	NA	
[6]SOFA	10;[5-16]	13;[9-17]		10;[2-12]	NA	
[4]CRP (mg/L)	264;[31-406]	151;[68-292]		85;[27-411]	29;[1-148]	
Leukocytes, $\times 10^9$/L	16;[22-90]	9;[4-36]		10;[5-27]	7;[4-22]	
Platelets, $\times 10^9$/L	199;[70-653]	247;[99-581]		286;[129-635]	334;[173-602]	
Lactate, mM	1.3;[0.7-4.8]	1.8;[0.7-5.6]		1.7;[0.4-6.6]	1.3;[1.0-2.6]	
Procalcitonin, µg/L	1.7;[0.2-79.5]	0.6;[0.1-66.1]		0.6;[0.1-25]	0.1;[0-1]	
D-dimer, mg/L	NA	4; [1-35]		NA	NA	
Fibrinogen, g/L	4.0 [3.2-9.0]	8.0; [4.0-9.0]		NA	NA	
Anticoagulant						
Heparin, n	15	14		8	7	
[8]PTT, s	38;[25-59]	36;[26-55]		41;[30-67]	34;[30-60]	

[1]ARDS, acute respiratory distress syndrome; [2]COVID-19, coronavirus disease 2019; [3]CRB 65, confusion, respiratory rate, blood pressure, age 65 score; [5]LOS ICU, length of intensive care unit stay, ; [5]ECMO, extracorporeal membrane oxygenation; [6]SOFA, sequential organ failure assessment; [7]CRP, C-reactive protein;[8]PTT, partial thromboplastin time.

Table 2. Baseline demographics and clinical characteristics of donors, COVID-19 and ARDS-influenza patients (lung tissue).

[1]COVID-19					
Patient	Age, year	Sex	Background	Ventilation, days	Anticoagulant
1	82	Male	Diffuse alveolar damage	0	Heparin
2	77	Male	Diffuse alveolar damage	2	Heparin
3	72	Male	Diffuse alveolar damage	6	Heparin
4	65	Male	Diffuse alveolar damage	33	Heparin
5	79	Female	Diffuse alveolar damage	2	Heparin
[2]ARDS-influenza					
1	67	Male	[3]CAP	6	Heparin
2	72	Male	CAP	10	Heparin
3	77	Male	CAP	5	Heparin
4	81	Female	CAP	10	Heparin
5	80	Female	CAP	3	Heparin
Donor					
1	82	Female	Recurrent myocardial infarction	0	no
2	75	Female	Heart and lung failure	0	Heparin
3	64	Male	Myocardial infarction	0	Heparin
4	77	Female	Dilated cardiomyopathy (right)	0	Heparin
5	75	Male	Dilated cardiomyopathy (right)	0	Heparin

[1]COVID-19, coronavirus disease 2019; [2]ARDS, acute respiratory distress syndrome; [3]CAP, community-acquired pneumonia

Figure 1. Activation of the contact phase system in plasma of critically ill COVID-19 patients. A,C) Western blot analysis (left panels) of factor XII (FXII) A) and high molecular weight kininogen (HK) C) in plasma from moderate and severe COVID-19 patients (infected with SARS-CoV2) and donors (healthy subjects). Four out of 15 moderate and severe COVID-19 patients and 3 out of 15 donors are demonstrated. Albumin was used as a loading control. Rights panels show the specificity of the antibodies used. B, D) Densitometric analysis of A) and C), respectively. COVID-19 moderate/severe n=15, donor n=15. E) PKa-like activity in plasma from moderate (n=14) and severe (n=14) COVID-19 patients and donors (n=15). F) Correlation between the levels of intact HK and PKa-like activity in plasma of severe Covid-19 patients. n=14. Correlation is performed using Spearman's rank correlation coefficient. *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Formation of dense fibrin clots in plasma of severe COVID-19. A) Western blot analysis of factor XII (FXII) in plasma from ARDS-influenza (Influenza) and COVID-19 (SARS-CoV2) patients as well as donors. Four out of 21 COVID-19 patients, 4 out of 25 ARDS-influenza patients, and 3 out of 21 donors are demonstrated. Albumin was used as a loading control. B) FXII levels in plasma of ARDS-influenza (n=25) and COVID-19 (n=21) patients as well as donors (n=16) as assessed by ELISA. C) Lag phase in fibrin formation-triggered by kaolin. Influenza, n=19; SARS-CoV2, n=20; donor, n=20. D) FVIII activity (FVIII:C) in plasma of patients and donors. Influenza, n=19; SARS-CoV2, n=20; donor, n=20. E, F) Time to reach the turbidity peak E) and maximum (Max) turbidity F) values for Influenza (n=19), SARS-CoV2 (n=20) and donor (n=20) plasma. Clot formation was induced by the addition of kaolin to plasma. G) Laser scanning confocal microscopy images of fibrin fibers in clots formed from Influenza (n=19), SARS-CoV2 (n=20), and donor (n=20) plasma. Representative pictures are demonstrated. H) Scanning electron microscopy images of fibrin network in clots generated from Influenza (n=5) as well as low- and high-fibrinogen SARS-CoV2 (n=5/group) plasma. Representative pictures are demonstrated. I) Fibrin fiber density in donor (n=20), ARDS-Influenza (n=19) and COVID-19 (n=20) clots. Per patient 3 separate clots were prepared, 5 pictures were taken in different areas of the clots and fibril density was determined in all pictures. J) Correlation between Max turbidity values and fibrinogen levels in plasma of COVID-19 patients (n=15; COVID-19 patients with available fibrinogen

levels were included into the analysis). Correlation is performed using Spearman's rank correlation coefficient.

Figure 3. Impact of FXIIa on fibrin clot structure in severe COVID-19 plasma. A, B) Max turbidity values of fibrin clots generated in the purified system from increasing concentrations of fibrinogen and/or Factor XII (FXII)/active FXII (FXIIa) in the absence or presence of corn trypsin inhibitor (CTI). Clot formation was induced by thrombin. n=4-5. C) Laser scanning confocal microscopy images of fibrin fibers in clots formed from FXII-depleted SARS-CoV2 or Influenza plasma supplemented with FXII. Representative pictures are shown. D) Fibrin fiber density in ARDS-Influenza (n=10) and COVID-19 (n=10) clots generated in C). Per patient 3 separate clots were prepared, 5 pictures were taken in different areas of the clots and fibril density was determined in all pictures. Paired data is shown interconnected. E) Rate of FXII (auto)-activation in ARDS-Influenza and SARS-CoV2 plasma. FXII was added to FXII-depleted plasma and its decay was monitored by western blotting using an antibody directed against FXII. Representative blot is shown. F) Quantification of FXII decay in ARDS-Influenza and SARS-CoV2 plasma in E. FXII signal at time point 0 was considered as 100%. n=20/group. G) Max turbidity values of fibrin clots generated by the addition of batroxobin to hirudin-preincubated plasma in the presence of FXIIa and/or CTI. n=15 biological replicates. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Dysregulated fibrinolysis in severe COVID-19. A) Turbidimetric analysis of clot lysis in severe COVID-19, ARDS-influenza and donor plasma. Representative clot lysis curves are shown. SARS-CoV2, n=20; ARDS-Influenza; n=19, donors, n=20. B) Turbidity values of the fibrin clots at 60 min. SARS-CoV2, n=20; ARDS-influenza, n=19; donors, n=20. C, D) Clot lysis time. Clots were generated in purified system with increasing concentrations of fibrinogen and/or factor XII (FXII)/active FXII (FXIIa). Clot formation was induced by thrombin and clot lysis by plasmin generated from plasminogen by tissue-plasminogen activator (t-PA). In some experiments FXII was preincubated with corn trypsin inhibitor (CTI). Clot formation and lysis were monitored *via* turbidimetry. n=3-5. *p<0.05, **p<0.01. E-G) t-PA (E), plasminogen activator inhibitor-1 (PAI-1; F), and thrombin-activatable fibrinolysis inhibitor (TAFI, G) levels in plasma of COVID-19 (n=21), ARDS-influenza (n=21) and donors (n=17) as assessed by ELISA.

Figure 5. High abundance of fibrin deposits in the lungs of severe COVID-19 patients. A, B) Fibrin (red) accumulation in postmortem lung tissue sections of severe COVID-19, ARDS-influenza, and donors (n=5/group). Time from death to autopsy was matched for all groups examined. Magnification bar 100 µm. B shows randomly chosen high magnification images of COVID-19 and ARDS-influenza clots presented in A. Arrows indicate fibrin deposits. All patients available are demonstrated. C) Fibrin abundance in COVID-19, ARDS-Influenza, and donor lungs. Per patient 5 pictures were taken in different areas of the lung and % of total area was determined in all pictures. n=5/group. *$p<0.05$, **$p<0.01$. D) Fibrin fiber density in COVID-19 and ARDS-Influenza lungs. Per patient 5 images of fibrin deposits were taken and fibril density was determined in all pictures. n=5/group.